

Conjugation of Mannose 6-Phosphate-containing Oligosaccharides to Acid α -Glucosidase Improves the Clearance of Glycogen in Pompe Mice*

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Clinical studies of enzyme replacement therapy for Pompe disease have indicated that relatively high doses of recombinant human acid α -glucosidase (rhGAA) may be required to reduce the abnormal glycogen storage in cardiac and skeletal muscles. This may be because of inefficient cation-independent mannose 6-phosphate receptor (CI-MPR)-mediated endocytosis of the enzyme by the affected target cells. To address this possibility, we examined whether the addition of a high affinity ligand to rhGAA would improve its delivery to these cells. Chemical conjugation of high mannose oligosaccharides harboring mono- and bisphosphorylated mannose 6-phosphates onto rhGAA (neo-rhGAA) significantly improved its uptake characteristics by muscle cells *in vitro*. Infusion of neo-rhGAA into Pompe mice also resulted in greater delivery of the enzyme to muscle tissues when compared with the unmodified enzyme. Importantly, this increase in enzyme levels was associated with significantly improved clearance of glycogen (~5-fold) from the affected tissues. These results suggest that CI-MPR-mediated endocytosis of rhGAA is an important pathway by which the enzyme is delivered to the affected lysosomes of Pompe muscle cells. Hence, the generation of rhGAA containing high affinity ligands for the CI-MPR represents a strategy by which the potency of rhGAA and therefore the clinical efficacy of enzyme replacement therapy for Pompe disease may be improved.

Pompe disease (glycogen storage disease type II), is an inherited disorder of glycogen metabolism caused by a deficiency of the lysosomal enzyme acid α -glucosidase (GAA)¹ (1, 2). The deficiency of GAA results in the lysosomal accumulation of glycogen in multiple tissues, with the cardiac and skeletal muscles being most severely affected. Pompe disease manifests as a broad spectrum of clinical severity and course that correlates with the extent of enzyme deficiency (3, 4). This disorder has been arbitrarily classified with designations based on the age of onset of symptoms, extent of organ involvement, and rate of progression to death. The infantile form of the disease is uniformly lethal and is characterized by a near total lack of

GAA activity and early onset of disease manifestations such as hypotonia, generalized muscle weakness, and profound hypertrophic cardiomyopathy. Patients with the infantile form of the disease invariably die around 1 year of age from cardiac failure (5). The juvenile and adult onset forms of Pompe disease present with residual enzyme activity, showing clinical symptoms at later ages, and generally exhibit more moderate cardiac involvement (6–10). However, progressive deterioration of the respiratory and skeletal muscles with age can lead to significant morbidity and in many instances early mortality, generally from respiratory failure. The disease is panethnic and has a combined incidence of ~1:40,000 live births (11, 12).

Over the past few years, significant efforts have been expanded to develop an enzyme replacement therapy (ERT) for Pompe disease (13). Recombinant human GAA (rhGAA) has been produced from a variety of sources such as in the milk of transgenic mice (14, 15) and rabbits (16), as well as from Chinese hamster ovary cells (17, 18). Preclinical studies in animal models of Pompe disease demonstrated that repeated intravenous infusions of these rhGAAs were safe and resulted in a near complete clearance of glycogen in cardiac muscles and a reduction (albeit incomplete) in the levels in the skeletal muscle (15, 16, 19, 20). Complete clearance of glycogen in skeletal muscle was not achieved even at the extremely high dose of 100 mg/kg. Subsequent clinical studies in infantile Pompe subjects also showed that ERT was effective in clearing glycogen storage from the heart but to a lesser degree from the skeletal muscles. Associated with this reduction in lysosomal storage was an improvement in muscle function, reversal of the hypertrophic cardiomyopathy, and an increase in the longevity of a proportion of the patients (21–24). However, the doses of rhGAA used in these clinical studies were relatively high, requiring 10–40 mg/kg/week, with partial clearance from skeletal muscle realized in some patients only at the 40 mg/kg/week dose (24). This is in contrast to the lower amounts of the respective lysosomal enzymes used in ERT of Gaucher, Fabry, and mucopolysaccharidosis type I patients, which typically range between 0.5 and 1 mg/kg, given biweekly (25, 26).

The basis for the requirement of higher levels of rhGAA, particularly to treat the skeletal muscle, is unclear but may be related to the low density of the cation-independent mannose 6-phosphate receptor (CI-MPR) that is present in this muscle (20, 27, 28). The CI-MPR-mediated pathway has been implicated as a major route for the internalization of rhGAA by cells *in vitro* and *in vivo* (1, 29). Uptake by Pompe cells *in vitro* is significantly inhibited by the addition of excess mannose 6-phosphate (M6P), indicating that the majority of the enzyme is internalized via this pathway (30). This receptor likely also has an important role for internalizing rhGAA and other lyso-

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¹ The abbreviations used are: GAA, acid α -glucosidase; ERT, enzyme replacement therapy; rhGAA, recombinant human acid α -glucosidase; CI-MPR, cation-independent M6P receptor; M6P, mannose 6-phosphate.

somal enzymes *in vivo*. For example, a recent study with another lysosomal enzyme (β -glucuronidase) indicated that greater efficacy at reducing the storage in the affected tissues of the mucopolysaccharidosis VII knock-out mice could be realized with a mannose 6-phosphorylated enzyme than with a non-phosphorylated enzyme (31).

Another possible reason for the requirement of higher doses of rhGAA may be related to the relatively low affinity of the enzyme for the CI-MPR. The possibility that the affinity of rhGAA for the receptor may be less than optimal has been suggested from experiments showing that efficient uptake of the enzyme by Pompe cells, at least *in vitro*, requires the presence of high concentrations of the enzyme in the growth media. To address this potential limitation, high affinity ligands (phosphorylated oligomannose-containing oligosaccharides) were prepared and chemically conjugated onto the oligosaccharide side chains of rhGAA. We showed that the modified rhGAA (neo-rhGAA) containing a higher content of M6P residues displayed improved uptake characteristics into myoblasts *in vitro* and facilitated greater clearance of glycogen from the muscles of Pompe mice. These observations are consistent with the importance of the CI-MPR in lysosomal enzyme trafficking and support the continued evaluation of similar approaches to improve the targeting of the enzyme to Pompe-affected tissues.

EXPERIMENTAL PROCEDURES

Materials—Phosphomannan isolated from yeast *Hansenula holstii* was a kind gift from Dr. Slodiki (32) and was hydrolyzed to phosphopentamannose according to the method of Bretthauer *et al.* (33). Phosphopentamannose was coupled to aminopropyl-agarose by reductive amidation according to the procedure of Distler *et al.* (34).

Isolation and Derivatization of M6P-containing Oligosaccharides—M6P-containing oligosaccharides were released from recombinant human α -galactosidase A (Genzyme Corp.) by digesting with endoglycosidase Hf and purified according to the method of Varki and Kornfeld (35) with minor modifications. The dialyzed oligosaccharides were adjusted to 2 mM Tris and then loaded onto a 20-ml QAE-Sephadex A column that had been equilibrated with the same buffer at a flow rate of 1.5 ml/min. The column was washed sequentially with 2 mM Tris containing 20 mM and 70 mM NaCl, and the M6P-containing oligosaccharides were then eluted with 2 mM Tris containing 200 mM NaCl. The purified M6P-containing oligosaccharides and the phosphopentamannose were derivatized to glycosylhydrazines using the method of Tolvanen and Gahmberg (36).

Chemical Conjugation of Derivatized M6P-containing Oligosaccharides onto rhGAA—Recombinant human α -glucosidase was dialyzed twice against 2 liters of 0.1 M sodium acetate (pH 5.6) for 18 h at 4 °C. The dialyzed rhGAA (5 mg/ml) was oxidized with 2 mM sodium metaperiodate for 30 min on ice. Excess sodium metaperiodate was consumed by the addition of 0.5 ml of 50% glycerol and by incubating on ice for 15 min. The oxidized enzyme was then dialyzed against 2 liters of 0.1 M sodium acetate (pH 5.6). Fifty-milligram aliquots of the oxidized rhGAA were conjugated to the hydrazine-derivatized M6P-containing oligosaccharides and phosphopentamannose (10 mg) by mixing and incubating at 37 °C for 2 h. After conjugation, both the M6P- and phosphopentamannose-conjugated rhGAA samples were dialyzed against 4 liters of 25 mM sodium phosphate buffer (pH 6.75) containing 1% mannitol and 0.005% Tween 80 for 18 h at 4 °C and then filtered through a 0.22- μ m filter (Whatman). The samples were aliquoted, snap-frozen on dry ice, and stored at -80 °C until used.

Cation-independent Mannose 6-Phosphate Receptor Column Binding Studies and Carbohydrate Analysis—Soluble CI-MPR was purified from fetal bovine serum using a phosphopentamannose column according to the method of Li *et al.* (37). The purified CI-MPR (1 mg) was coupled to 1-ml Affi-Gel-15 beads (Bio-Rad) essentially as outlined by the manufacturer. Binding of rhGAA or M6P-conjugated rhGAA to the CI-MPR column was performed as described by Valenzano *et al.* (38). The M6P contents of rhGAA and neo-rhGAA were analyzed using the method described by Zhou *et al.* (39). Oligosaccharide profiling of the purified M6P-containing oligosaccharides following endoglycosidase Hf digestion of α -galactosidase A was performed according to the method of Townsend and Hardy (40).

In Vitro Cell Uptake Studies—L6 myoblast cells (ATCC) were plated onto 12-well culture dishes and allowed to settle for 24 h. Prior to the

addition of enzyme, the cells were washed once with 3 ml of Dulbecco's modified Eagle's medium. Various forms of rhGAAs in 1 ml of uptake medium (Dulbecco's modified Eagle's medium containing 1% (v/v) heat-inactivated fetal bovine serum, 25 mM Hepes (pH 6.8), 2.5 mM β -glycerophosphate, and antibiotics) were then added and incubated at 37 °C for 18 h. In some of the wells, 5 mM M6P was added to inhibit CI-MPR-mediated uptake. After 18 h, the cells were washed twice with phosphate-buffered saline containing 1 mM M6P and then twice more with phosphate-buffered saline alone. The cells were lysed in GAA assay buffer (0.2 M sodium acetate, 0.4 M potassium chloride, pH 4.3) containing 0.1% Triton X-100 by scraping followed by sonication. The lysates were centrifuged at 14,000 $\times g$ for 10 min at 4 °C, and the GAA activity in the cleared supernatants was assayed using the fluorogenic substrate 4-methylumbelliferyl- α -D-glucopyranoside (17). The protein content in the cell lysates was determined using the microBCA kit (Pierce) with bovine serum albumin as a standard. GAA activity in Pompe mice tissues was assayed using the same method except that the assay buffer contained 0.5% Triton X-100.

Animal Studies—Animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services, NIH Publication 86-23). Four- to five-month-old Pompe mice (41) were used to evaluate the relative ability of various rhGAAs to reduce the glycogen storage in the affected tissues. Groups of Pompe mice (seven animals/group) were injected via the tail vein with vehicle and varying doses of rhGAA or neo-rhGAA. The mice were administered three weekly doses and were then sacrificed 2 weeks after the last treatment. Various tissues including the heart, diaphragm, and skeletal muscle were collected and stored at -80 °C until assayed. Statistical analysis was performed using one-way analysis of variance followed by a Newman-Keuls test. A probability value of $p < 0.05$ was considered statistically significant.

Measurement of Glycogen Levels—The glycogen content in the various muscles of the Pompe mice was assayed by measuring the difference in the amount of glucose released from a boiled tissue homogenate following digestion with or without *Aspergillus niger* amyloglucosidase as described previously (42). The glucose levels were assayed using the Amplex Red glucose assay kit (Molecular Probes) according to the manufacturer's instructions. Bovine liver glycogen was used as a standard. In some studies, glycogen content was measured using periodic acid-Schiff staining followed by computer-assisted histomorphometric analysis (MetaMorph) as described previously (20). All photography and MetaMorph analyses were performed in a blinded manner.

RESULTS

Process for Chemically Conjugating M6P-containing Oligosaccharides onto rhGAA Did Not Affect Its Enzymatic Activity—Direct chemical conjugations of oligosaccharides onto a protein backbone via reductive amination or maleimide chemistries frequently require prolonged incubations at neutral to alkaline pH. These reaction conditions are destabilizing to lysosomal enzymes such as GAA that have optimal activities at acidic pH. To minimize the inactivation of these enzymes, a conjugation scheme was used that employed a condensation reaction between an aldehyde group and a hydrazine to form a hydrazone bond. In this scheme, M6P-containing oligosaccharides were derivatized to glycosylhydrazines and then conjugated (at acidic pH) to rhGAA, the sialic acids of which had been oxidized with periodate to aldehydes. Conjugating the M6P-containing moieties directly onto the existing oligosaccharide side chains of rhGAA also confers spacer length that could minimize the effect of steric hindrance during receptor binding.

Using phosphopentamannose-hydrazine as a model ligand, we demonstrated that its conjugation onto rhGAA did not affect the hydrolytic activity of the enzyme (data not shown). Conjugation efficiency was determined to be high and to have occurred on nearly all rhGAA as evidenced by an increase in the binding of the phosphopentamannose-conjugated rhGAA to a CI-MPR column (Fig. 1). Although only ~40% of the original rhGAA bound the CI-MPR column, the column retained more than 90% of the phosphopentamannose-conjugated rhGAA. Hence, the conjugation process used to modify the oligosaccharides on rhGAA was efficient and did not measurably alter its

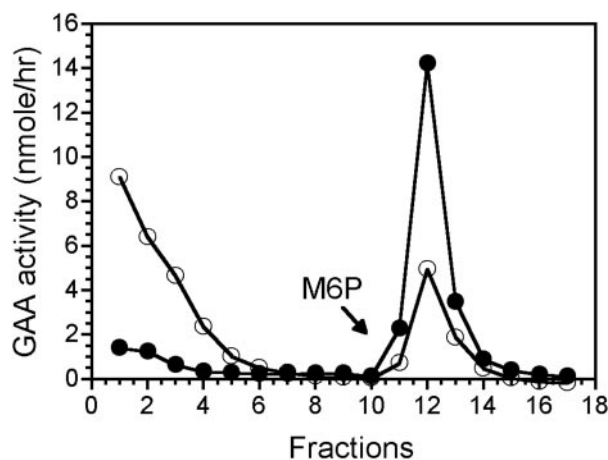


FIG. 1. Chromatography of rhGAA (open circles) and phosphopentamannose-conjugated rhGAA (closed circles) over a CI-MPR column. Approximately 10 μ g of each of the enzymes was loaded onto a 2-ml CI-MPR column. After washing the column with binding buffer, the bound material was eluted (starting at fraction 11) with binding buffer containing 5 mM M6P. Two-ml fractions were collected and assayed for GAA activity.

activity. However, conjugation with phosphopentamannose did not enhance its uptake into L6 myoblasts *in vitro* when compared with the unmodified enzyme (data not shown). This may be expected because phosphopentamannose is a relatively low affinity ligand for the CI-MPR (34).

Conjugation of Mono- and Bisphosphorylated Oligomannose Residues onto rhGAA Improved Its Binding to CI-MPR and Uptake into Cells *in Vitro*—To generate a modified rhGAA with a higher affinity ligand for the CI-MPR, M6P-containing oligosaccharides were isolated from recombinant human α -galactosidase A and conjugated onto rhGAA using the same scheme. Recombinant α -galactosidase A was used as a source of the oligosaccharides because analysis of its carbohydrate (Fig. 2a) indicated that 30–40% of the high mannose oligosaccharides are bisphosphorylated, a high affinity ligand for the CI-MPR. Phosphorylated high mannose oligosaccharides (both mono- and bisphosphorylated) were released from α -galactosidase A by endoglycosidase H treatment and purified over a QAE column. Conjugation of the purified mono- and bisphosphorylated oligosaccharides onto rhGAA (neo-rhGAA) resulted in an increase in the fraction of enzyme that bound to the CI-MPR column (Fig. 2b). Approximately 63% of the neo-rhGAA bound the CI-MPR column compared with \sim 40% for the unmodified enzyme. The lower than expected fraction of neo-rhGAA (63% as opposed to \geq 90% for phosphopentamannose-conjugated rhGAA) that bound the CI-MPR column was because smaller amounts of the phosphorylated oligosaccharides were used in the conjugation reaction. The increased binding of neo-rhGAA was not because of nonspecific interactions between the oxidized sialic acids on rhGAA and the CI-MPR column because periodate-treated but non-conjugated rhGAA displayed binding characteristics that were similar to those for untreated rhGAA (Fig. 2b).

Monosaccharide analysis of the neo-rhGAA confirmed that the modified enzyme contained higher levels of phosphorylated oligomannose residues. The M6P content was increased from 0.9 mol M6P/mol for the unmodified rhGAA to 2.9 mol M6P/mol for the neo-rhGAA. Importantly, this increase in M6P-containing oligosaccharides on neo-rhGAA resulted in a significant enhancement in its uptake by L6 myoblasts (Fig. 3). Uptake of neo-rhGAA approached saturation at 100 nM compared with \sim 500 nM for the unmodified rhGAA. This is consistent with an increase in the affinity of neo-rhGAA for the CI-MPR, presum-

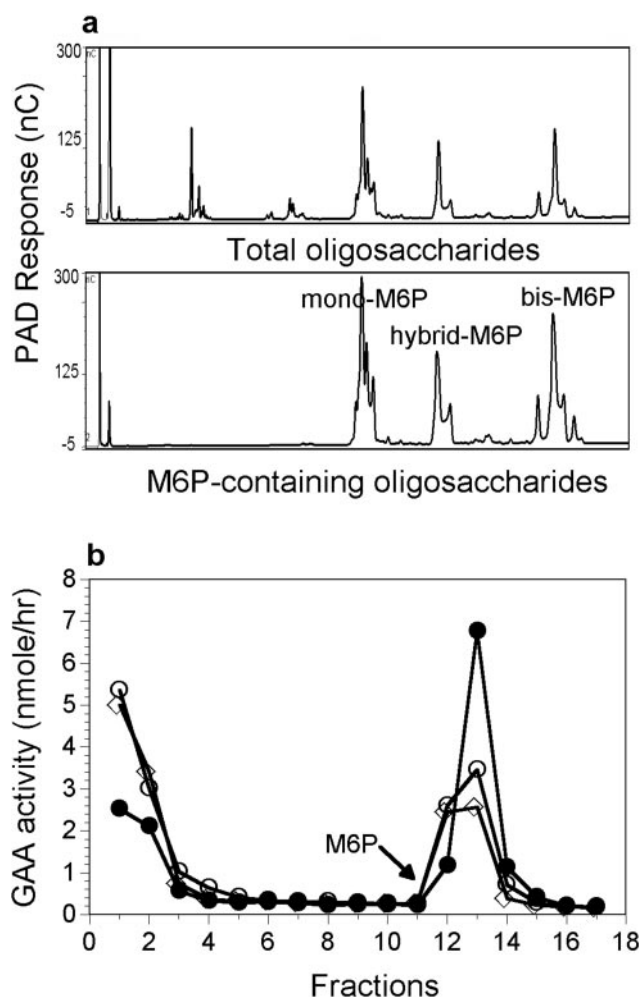


FIG. 2. a, analysis of the oligosaccharides released following digestion of α -galactosidase A with endoglycosidase H (upper panel) and following purification of the M6P-containing fraction over a QAE column (lower panel) by Dionex column chromatography. b, chromatography of rhGAA (open circles), neo-rhGAA (closed circles), and periodate-treated rhGAA (open squares) over a CI-MPR column as described in the legend for Fig. 1. PAD, pulsed amperometric detection.

ably because of the conjugation of additional M6P-containing ligands. Uptake was completely blocked by the addition of excess M6P, confirming that the uptake of the enzyme by the L6 cells was primarily mediated via the CI-MPR (data not shown).

Clearance of Glycogen from Pompe Mouse Tissues Was Improved with Neo-rhGAA—To determine whether the improved uptake characteristics of neo-rhGAA *in vitro* would lead to a greater reduction in glycogen storage *in vivo*, 4–5-month old Pompe mice were treated with either neo-rhGAA or unmodified rhGAA. Mice were administered three weekly doses and were killed 2 weeks after the last treatment. Approximately 24 and 46% higher enzyme levels were detected in the skeletal muscle and heart, respectively, of animals administered neo-rhGAA when compared with those treated with unmodified rhGAA. Treatment with either form of the enzyme resulted in a dose-dependent reduction in the glycogen levels (Fig. 4). At equivalent doses, mice treated with neo-rhGAA uniformly displayed a greater extent of glycogen reduction in all the muscles analyzed (Fig. 4). In the heart, a \sim 4–6-fold greater reduction in glycogen levels was attained with neo-rhGAA than with rhGAA at both the 10 and 20 mg/kg doses. Significantly higher reductions in glycogen levels were also observed in the other muscle tissues of animals that had been treated with the modified enzyme

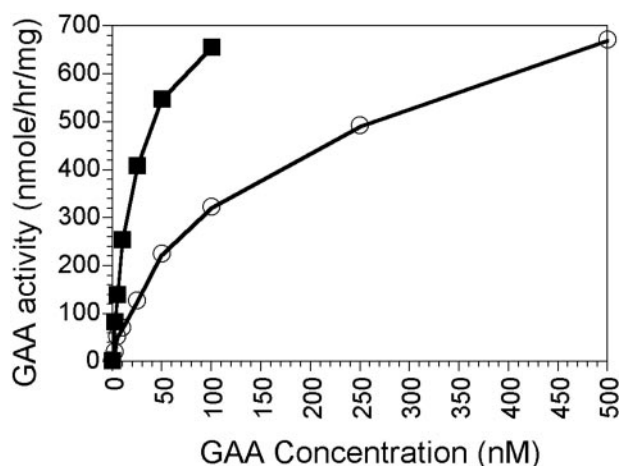


FIG. 3. *In vitro* uptake of neo-rhGAA into L6 myoblasts. Increasing amounts of either rhGAA (open circles) or neo-rhGAA (closed squares) were added to L6 myoblasts and incubated at 37 °C for 18 h. The cells were then washed and harvested, and the lysates were assayed for GAA activity. Endogenous GAA levels were subtracted from the data presented.

(Fig. 4). An ~50% reduction in glycogen was attained with neo-rhGAA in the quadriceps muscles and to lesser extents in the triceps and diaphragm. In the quadriceps, treatment with unmodified rhGAA had no effect. In nearly all cases, the efficacy attained with only 20 mg/kg neo-rhGAA was similar to that achieved with 50 mg/kg unmodified rhGAA (Fig. 4). Consistent with previous data (20, 43), the skeletal muscles were more refractory to treatment than the heart even with neo-rhGAA, attaining a 50–60% reduction in glycogen at the 20 mg/kg dose compared with 95% for the heart (Fig. 4).

The reduction in glycogen levels observed by biochemical analysis was confirmed by histomorphometric assessment of the quadriceps muscles obtained from the same animals (Fig. 5). Lysosomal glycogen following staining and analysis of the tissue samples by high resolution light microscopy appeared as discreet, purple-beaded structures scattered throughout each myocyte (Fig. 5a). With enzyme treatment, these glycogen-containing structures became smaller and fewer in number. The administration of 20 mg/kg neo-rhGAA resulted in a 54% reduction in the tissue area occupied by glycogen when compared with the vehicle-treated samples (Fig. 5b). This reduction was nearly as effective as the administration of 50 mg/kg unmodified rhGAA, which provided for a 60% reduction, suggesting that neo-rhGAA was 2–2.5 times more potent than rhGAA. Treatment with 10 mg/kg rhGAA resulted in a 17% reduction in glycogen (Fig. 5b). Because the extent of glycogen reduction at this dose was similar to that attained with 20 mg/kg rhGAA (Fig. 4), comparison of this result with that of 20 mg/kg neo-rhGAA (54% reduction) would indicate an ~3-fold increase in potency with neo-rhGAA.

DISCUSSION

The success of ERT for Pompe disease is dependent on the ability to deliver exogenously administered rhGAA to all of the affected tissues and to a level that is sufficient to effect a net reduction in lysosomal storage of glycogen. Because Chinese hamster ovary-derived rhGAA contains a mixture of high mannose and complex-type oligosaccharide side chains, uptake of the enzyme from the circulation is likely mediated by a number of cell surface receptors including the cation-independent mannose 6-phosphate, asialoglycoprotein, and mannose receptors. Although the relative contributions of the different receptors remains unclear, there is evidence that the CI-MPR plays a significant role in the uptake of rhGAA and, indeed, the ma-

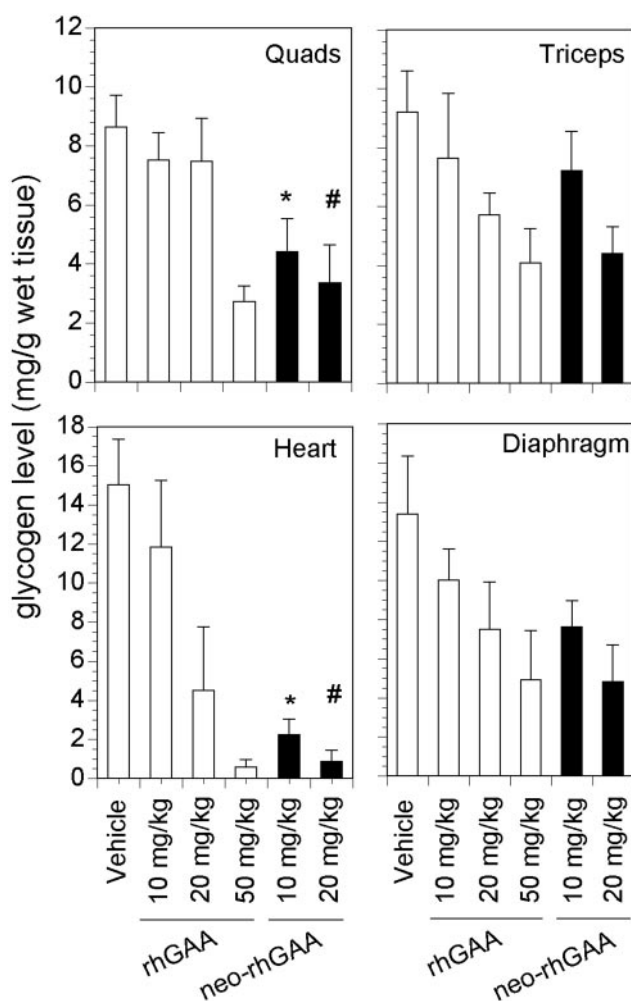


FIG. 4. Glycogen levels following treatment with unmodified and modified rhGAA. Pompe mice were administered increasing amounts of either rhGAA or neo-rhGAA. The mice were treated with three weekly doses of the enzymes and killed 2 weeks after the last treatment. Tissues were collected and assayed for glycogen using the Amplex Red glucose assay. The results are representative of two independent experiments with seven animals in each group. * and #, $p < 0.001$ in comparison with the 10 mg/kg rhGAA and 20 mg/kg rhGAA groups, respectively.

jority of the different lysosomal enzymes. For example, greater uptake of GAA by the heart and skeletal muscles was realized with an enzyme preparation that contained mannose 6-phosphorylated residues than one lacking this moiety (29). Improved efficacy in treating another lysosomal storage disease animal model (mucopolysaccharidosis VII) was also observed when a highly mannose 6-phosphorylated β -glucuronidase was used in lieu of a non-phosphorylated preparation (31). However, the abundance of the CI-MPR is relatively low in skeletal muscles, which may explain the low level of enzyme uptake by this tissue (20, 27, 28). This problem may also be exacerbated by the fact that delivery to the muscle fibers requires that the enzyme traverse not only the endothelial barrier but also the endomysium. The combined poor accessibility of the skeletal muscle fibers to systemically delivered rhGAA and the low abundance of the CI-MPR could account for the reported requirement of high doses of enzyme to treat this tissue (20, 24).

Given these considerations, several strategies could be envisaged to improve the treatment of Pompe-affected tissues. For example, increasing the potency of the enzyme by generating a modified GAA with higher specific activity or longer

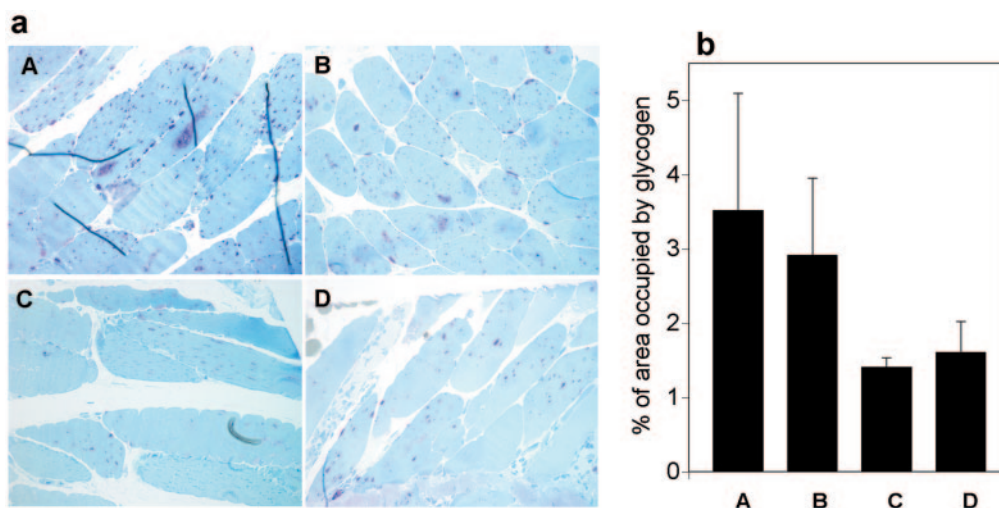


FIG. 5. Glycogen was visualized in quadriceps muscle samples by high resolution light microscopy and measured using computer-assisted histomorphometry. *a*, glycogen was visualized as purple-beaded structures within the myocytes. *b*, the glycogen content in the samples were quantitated and expressed as percent tissue area occupied by glycogen. A reduction in glycogen was readily apparent in samples treated with either 50 mg/kg rhGAA or 20 mg/kg neo-rhGAA when compared with the vehicle control. A, vehicle control; B, 10 mg/kg rhGAA; C, 50 mg/kg rhGAA; D, 20 mg/kg neo-rhGAA.

half-life may allow for delivery of smaller amounts of the enzyme for therapeutic efficacy. Another strategy is to improve the targeting of the enzyme to the affected tissues. This could be realized either by increasing the abundance of the CI-MPR at the cell surface or the affinity of the recombinant enzyme for the receptor(s). Characterization of the Chinese hamster ovary-derived rhGAA used in this study indicated that it had a relatively low affinity for the CI-MPR, at least when tested *in vitro*. Uptake of the enzyme by L6 myoblasts showed saturation at around 500 nM rhGAA. This approximates the dissociation constant (K_d) reported for a monophosphorylated oligosaccharide for the CI-MPR (34, 44), suggesting that the rhGAA was composed predominantly of this low affinity ligand. The addition of more M6P moieties onto rhGAA to generate neo-rhGAA increased its uptake into myoblasts *in vitro*. Uptake of neo-rhGAA was saturated at 50–100 nM. The higher than predicted K_d value observed with neo-rhGAA likely reflected the fact that both mono- and bisphosphorylated oligosaccharides were conjugated onto the enzyme. Additionally, it is also likely that not all of the rhGAA were successfully conjugated with the phosphorylated oligosaccharides. Based on the fraction of enzyme that bound the CI-MPR column prior to and following conjugation (Fig. 2*b*), it was estimated that only ~38% of the neo-rhGAA was successfully conjugated with bisphosphorylated mannose 6-phosphates. Incomplete conjugation would also contribute to the higher than expected apparent K_d of neo-rhGAA for the CI-MPR (50–100 nM), which if mediated solely by bisphosphorylated mannose 6-phosphates should be ~2 nM (44).

This modification of rhGAA to contain a higher affinity ligand for the CI-MPR was most likely responsible for the observed improved delivery of neo-rhGAA to the Pompe mouse muscles. Increased delivery of neo-rhGAA to the muscles was associated with greater clearance of glycogen from the tissues. After adjusting for the conjugation efficiency (38%), it was estimated that an improvement of ~5-fold in skeletal muscle and 10-fold in heart was realized with neo-rhGAA. However, it is anticipated that further improvements in efficacy should be attainable through the judicious use of synthetic glycans containing the optimal configuration of M6P residues (34, 45). Use of synthetic glycans in lieu of the oligosaccharides released from α -galactosidase A should eliminate the addition of the low affinity monophosphorylated oligosaccharides and also mini-

mize the number of free terminal mannose and galactose residues present on the oligosaccharides used for conjugation. The latter could have the effect of reducing the uptake of the modified enzyme by mannose and asialoglycoprotein receptors and thereby improve the pharmacokinetic profile of the drug. The improved efficacy associated with the use of such modified rhGAAs should in turn allow for a significant reduction in the dose required to clear the accumulated glycogen from the affected tissues. This is not a trivial benefit considering that significant clearance of glycogen from the skeletal muscle of Pompe mice treated with unmodified rhGAA was only observed at a dose of 50 mg/kg. Yet higher doses of rhGAA (100 mg/kg) were reportedly necessary if older Pompe animals (>6 months old) were used at the start of the study (20). The basis for the requirement of higher doses to treat older animals is unclear but may be related to the extent of muscle damage and the declining abundance of the CI-MPR in the muscle with age (28).

The data generated here supports the premise that the CI-MPR is involved in the uptake of a proportion of systemically delivered rhGAA, particularly by skeletal muscles. This is perhaps a not too surprising finding given the body of data reported in the literature thus far (29, 30). However, it remains possible that other receptors may also have a role in uptake of the enzyme, the extent of which remains to be determined. If that is correct, other ligands could be similarly exploited to augment the delivery of rhGAA to various Pompe-affected organs. A recent report using an insulin-like growth factor II-derived peptide to deliver β -glucuronidase is an example (47). The strategy of modifying the carbohydrate composition of an enzyme to improve its therapeutic effect is not without precedent. During the development of recombinant glucocerebrosidase for ERT of Gaucher disease, significantly improved efficacy was also realized after remodeling the carbohydrate on the enzyme to expose core mannose residues (25, 46). This modification facilitated greater binding of the enzyme to the mannose receptors present on affected Gaucher macrophages. The results here are consistent with these observations and argue that remodeling the carbohydrate of rhGAA, such that it becomes a higher affinity ligand for the CI-MPR or indeed for other receptors that may be present on the affected cells, represents a viable strategy to enhance the efficacy of ERT for Pompe disease.

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